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ance Notes on Codes and Abbreviations" appearing at the begin-
ning of each regular issue of the PCT Gazette.*

(54) Title: **DIAGNOSTIC TESTING PROCESS AND APPARATUS**

(57) Abstract: A method and apparatus for use in a flow through assay process is disclosed. The method is characterised by a "pre-incubation step" in which the sample which is to be analysed, (typically for the presence of a particular protein), and a detection analyte (typically an antibody bound to colloidal gold or a fluorescent tag) which is known to bind to the particular protein may bind together for a desired period of time. This pre incubation step occurs before the mixture of sample and detection analyte come into contact with a capture analyte bound to a membrane. The provision of the pre-incubation step has the effect of both improving the sensitivity of the assay and reducing the volume of sample required for an assay. An apparatus for carrying out the method is disclosed defining a pre-incubation chamber for receiving the sample and detection analyte having a base defined by a membrane and a second membrane to which a capture analyte is bound. In one version the pre-incubation chamber is supported above the second membrane in one position but can be pushed into contact with the membrane carrying the capture analyte thus permitting fluid transfer from the incubation chamber through the capture membrane. In another version the membrane at the base of the incubation chamber is hydrophobic and its underside contacts the capture membrane and when a wetting agent is applied to the contents of the pre-incubation chamber fluid transfer occurs.



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Diagnostic testing process and apparatus

Field of the Invention

This invention relates to a diagnostic testing process and in particular
5 to an apparatus for use in carrying out an assay process and to a method of carrying out an assay process using that apparatus.

Background of the Invention

Lateral flow and flow-through technology have been used for
10 diagnostic assays for almost twenty years. Lateral flow technology is currently dominant because lateral flow devices are easy to produce and the assay can be performed in a simple 2-step process that can be adapted for whole blood separation. This results in a simple device that can be used in the field as a rapid point-of-care diagnostic (Cole et al 1996 Tuberc. Lung. Dis.
15 77:363-368). However, multiple disease diagnosis using lateral flow technology is very difficult because of differences in lateral diffusion between samples and variation in flow rates between batches of the partitioning membrane. This means that antigen or antibody signal strengths may vary both within tests and between batches of tests, resulting in inconsistent
20 results.

Existing flow-through diagnostic tests can be completed in less than two minutes compared with typical times of five to fifteen minutes for lateral flow tests. This advantage in speed however, is often at the expense of sensitivity. A further disadvantage is that higher volumes of sample are
25 required to achieve the same sensitivity as lateral flow. This may be problematic in some situations. For example, the diagnosis of analytes (reagents) in whole blood requires the separation of plasma from whole blood cells. The higher volumes of whole blood required for this would quickly block the membranes in the flow-through format.

30 The basic principal of flow-through assays is well established. The tests are designed to determine the existence of, and in some cases, the quantity of, a predetermined analyte/reagent in a sample. Often the reagent will be a protein but other reagents can be tested for. If the assay is to test for the existence of a particular disease in a patient, the patient's body fluids may
35 be tested for an antibody or other protein produced by the patient in response to the infection, or for a protein which is expressed by the bacterium or viral

agent or the like causing the disease. In a typical flow through assay a liquid sample which is believed to contain the reagent is sucked into an absorbent pad via a membrane to which is bound a capture analyte which is known to bind to the reagent. The membrane is then typically washed with a buffer
5 and a liquid containing a detection analyte which also binds to the reagent and which includes a tracer or marker which is detectable, is applied to the membrane. The detection analyte binds to the immobilised reagent bound to the membrane and can be seen or otherwise detected to indicate the presence of the reagent.

10 US 4246339 discloses a test device for assaying liquid samples for the presence of a predetermined reagent. The device includes telescoping top and bottom members defining a liquid reservoir therebetween and resilient means for biasing the members in the open position. The top member defines a series of test wells each of which has a base defined by a microporous
15 membrane with a capture analyte immobilised on the membrane surface. Absorbent means are located in the bottom member, spaced from the membrane in the open position but in contact therewith in the closed position. US 4246339 discloses the adding the test serum diluted with a buffer to a test well, and incubating the device at room temperature for ten
20 minutes prior to depressing the cassette to the closed position to pass the sample through the membranes into the absorbent material. When the membranes are dry, the membrane is washed and then covered with a solution containing a detection analyte which binds to the immobilised reagent followed by a subsequent step in which a stain is applied.

25 It will be appreciated that the process described in US 4246339, is a somewhat long drawn out, time consuming and tedious process and also lacks sensitivity.

A more recent flow through device is described in US 5185127 which discloses an assay device including a filter stack and an enclosure having a
30 base portion and a lid. The filter stack has a hydrophilic membrane having a capture analyte thereon, referred to in US 5185127 as a binder. A hydrophobic membrane is located under the hydrophilic membrane and a pad of absorbent material is located under the hydrophobic membrane. The lid includes an upwardly extending rib which defines a recess having an
35 insert therein. In use, a sample containing the reagent (referred to in US 5185127 as the analyte) is placed in the well of the assay device at which

time the reagent/analyte binds to the capture analyte/binder. Flow of the assay solution however, does not take place because the aqueous solution does not wet the hydrophobic membrane placed under the hydrophilic membrane in the filter stack. Thus as much time is necessary to complete the binding of the detection analyte to the reagent is allowed. When binding is judged to be complete, flow may be initiated by adding a wetting agent which wets the hydrophobic membrane. After which time the aqueous liquid flows into pad of absorbent material. The membrane may then be washed and treated with a detection analyte/tracer which may be an antibody which specifically binds to the analyte, the antibody having a label covertly conjugated thereto. Again the sensitivity of US 5185127 is lacking and is not equivalent to that obtainable in lateral flow or ELISA formats.

It is one object of the present invention to provide an improved method and apparatus for use in an assay process such as an immunoassay, diagnostic assay or the like in which the process and apparatus are capable of screening a wide range of samples such as whole blood, plasma/serum, or samples with a high particulate load such as crushed grain (eg wheat heads) and which is simple and rapid to perform whilst still maintaining sensitivities at least equivalent to that obtainable in lateral flow or ELISA formats.

A related object of the present invention is to provide a method and apparatus which can be successfully used for multiple disease diagnosis from a single whole blood or other sample in a single test. An extension would be successful screening of a sample in a single test for the presence of multiple analytes not necessarily related to disease (e.g drugs, agriculture, veterinary testing).

Any discussion of documents, acts, materials, devices, articles or the like which has been included in the present specification is solely for the purpose of providing a context for the present invention. It is not to be taken as an admission that any or all of these matters form part of the prior art base or were common general knowledge in the field relevant to the present invention as it existed in Australia or elsewhere before the priority date of each claim of this application.

Because the prior art is not consistent in its terminology, for the avoidance of doubt and for the purpose of clarity, the following terms used in the specification below, are defined as follows. The term "reagent" is used to

refer to the compound protein or the like which is to be detected by the assay. The term "capture analyte" is used to refer to a compound which is bound to a membrane and to which the reagent will bind. The term "detection analyte" is used to refer to a compound which will also bind to the reagent and which
5 carries a tracer or some other element whose presence may be detected, typically visually detected whether under visible light, or fluorescence.

Summary of the Invention

In a first broad aspect, the present invention provides an apparatus and
10 method for use in an assay process which is characterised by providing a "pre-incubation step" in which the sample and detection analyte (which may typically be an antibody bound to colloidal gold or a fluorescent tag) may bind together, which has the effect of both improving the sensitivity of the assay and reducing the volume of sample required for an assay prior to
15 reaction of the sample/analyte complex with a reaction membrane to which one or more ligands are bound.

Thus, in one aspect of the present invention there is provided an apparatus for use in an assay process comprising:

a first member comprising a first, porous, reaction membrane to which
20 is bound a capture analyte for binding to a reagent to be detected, the member having an upper surface and a lower surface;

a second member being a body of absorbent material such as tissue paper or the like disposed below and touching the lower surface of the first member;

25 a chamber spaced above the first member said chamber having side walls, and a base defined by a second membrane; and

means for supporting the chamber above the first member in two positions, a first position in which the membrane is spaced a sufficient distance from the first member so as to not permit fluid transfer from the
30 chamber to the body of absorbent material, and a second position in which the membrane is in contact with the first member thus permitting fluid transfer from the chamber through the first and second membranes to the body of absorbent material.

In a related aspect the present invention provides a method for
35 assaying for the presence of a pre-determined reagent using an apparatus of the present invention comprising the steps of:

- a) placing a sample to be assayed and a detection analyte in the chamber, with the chamber disposed in the first position;
- b) allowing a sufficient period of time to pass for the detection analyte to bind to the reagent, if present;
- 5 c) depressing the chamber to the second position to contact the base of the chamber with the first porous membrane;
- d) allowing the sample to flow through the first and second membranes to allow the reagent, if present to bind to the capture analyte carried on the first membrane.

10 Thus, the present invention provides a chamber which may serve as a pre-incubation chamber in which a pre-incubation step can occur where the sample and detection analyte combine, which improves the sensitivity of the test and reduces the volume of sample required for the assay. It has been
15 found that the pre-incubation step increases the test sensitivity for a typical existing flow-through apparatus by approximately ten times to equivalent levels of sensitivity compared with lateral flow technology, while still allowing the assay to be completed in around two minutes compared to 10 minutes for lateral flow formats.

For example a ground wheat head suspension may be solubilised, and
20 mixed and pre-incubated in the chamber with a detection analyte in the form of an antibody against alpha-amylase linked to a colloidal gold particle. The contents of the chamber may then be allowed to flow through to the first membrane containing a capture analyte in the form of an immobilised anti-amylase antibody, and anti-amylase antibody/gold complexes will bind to the
25 immobilised antibody forming a detectable signal. The signal can be detected by the removal of the pre-incubation unit and washing of the reaction membrane with buffer.

This format can also be used for detecting reagents in whole blood since whole red blood cells can be removed in the pre-incubation chamber
30 and the plasma allowed to flow-through to the reaction membrane containing a bound capture analyte. In this format, the base membrane defined at the base of the pre-incubation chamber will typically be a membrane which has the correct pore size to retain the red blood cells and allow the plasma to pass through on contact with the first membrane. Similarly particulate samples
35 containing grain extracts, cell or microbial extracts can be analysed with this flow-through format since particulate matter can be removed in the pre-

incubation chamber and therefore cannot block the reaction area on the upper surface of the reaction membrane.

The apparatus can also be used for detecting analytes in body fluids other than blood, such as plasma, sera, urine, saliva and sputum. In this case, the sample can be retained in the pre-incubation chamber by use of a hydrophobic membrane. To obtain efficient flow through capillary action to the second member when the pre-incubation chamber is lowered, the reaction membrane is pre-wet with a wetting agent containing a detergent or the reaction membrane is blocked with a hygroscopic solution such as sucrose, trehalose, fructose, or alternatively, glycerol.

This changes the characteristics of the reaction membrane from a non-hygroscopic to a hygroscopic membrane allowing the sample to flow through to the second member upon contact of the membrane at the base of the pre-incubation chamber with the reaction membrane.

In a yet further embodiment, if a hydrophobic membrane is used as the base of the pre-incubation chamber, the apparatus may be used with the hydrophobic membrane and reaction membrane in contact, with the operator adding a wetting agent to the sample to cause flowthrough, when desired.

Thus, in a related aspect, there is provided an apparatus for use in an assay process comprising a housing including:

- a first member comprising a first, porous, membrane to which is bound a capture analyte for binding to a reagent to be detected, the member having an upper surface and a lower surface;

- a second member being a body of absorbent material such as tissue paper or the like disposed below and touching the lower surface of the first member;

- a chamber located above the first member said chamber having side walls, and a base including a second, hydrophobic, membrane, having an upper and a lower surface, the pre-incubation chamber being supported above the first member with the lower surface of the hydrophobic membrane in contact with the upper surface of the first member.

The pre-incubation chamber can also be used to remove analytes that may interfere with the assay, such as human anti-mouse antibodies (HAMAS), in solution or by binding anti-analyte antibodies to the surface of the chamber. The chamber can also be used to extract the analyte of interest from an absorbent surface such as a swab, which has been taken from the

throat of a patient, by swirling the swab in an extraction solution in the chamber. The pre-incubation chamber may be part of a pre-filter unit which acts also to pre-filter the sample prior to contact with the upper surface of the first member.

- 5 Examples of assays that can be preformed by this method where two reaction steps are involved (the incubation of the analyte with the labeled anti-analyte followed by the binding of this complex to a solid-phase anti-analyte), are:

10 Direct antigen assay

1. $Ag^* (analyte) + Ab^*_1 (anti-Ag)\text{-label}$
2. Solid phase- $Ab_2 (anti-Ag) + Ag/Ab_1(anti-Ag)\text{-label complex}$

Direct antibody assay (i)

- 15 1. $Ab_1 (analyte = anti-Ag) + Ab_2 (anti-Ab_1)\text{-label}$
2. Solid phase- $Ag + Ab_1 (anti-Ag)/Ab_2 (anti-Ab_1)\text{-label complex}$

Direct antibody assay (ii)

1. $Ab_1 (analyte = anti-Ag) + Ab_2 (anti-Ab_1)\text{-label}$
- 20 2. Solid-phase- $Ab_3 (anti-Ag)/Ag + Ab_1 (anti-Ag)/Ab_2 (anti-Ab_1)\text{-label complex}$

Indirect antigen assay

1. $Ag (analyte) + Ab_1 (anti-Ag) + Ab_2 (anti-Ab_1)\text{-label}$
- 25 2. Solid-phase- $Ab_3 (anti-Ag) + Ag/Ab_1 (anti-Ag)/Ab_2 (anti-Ab_1)\text{-label complex}$

Indirect antibody assay (i)

1. $Ab_1 (analyte = anti-Ag) + Ab_2 (anti-Ab_1) + Ab_3 (anti-Ab_2)\text{-label}$
- 30 2. Solid phase $Ag + Ab_1 (anti-Ag)/Ab_2 (anti-Ab_1)/Ab_3 (anti-Ab_2)\text{-label complex}$

Indirect antibody assay (ii)

1. $Ab_1 (analyte = anti-Ag) + Ab_2 (anti-Ab_1) + Ab_3 (anti-Ab_2)\text{-label}$
- 35 2. Solid phase $Ab_4 (anti-Ag)/Ag + Ab_1 (anti-Ag)/Ab_2 (anti-Ab_1)/Ab_3 (anti-Ab_2)\text{-label complex}$

*Ag indicates antigen

*Ab indicates antibody

5 A piezoelectric driven printer may be used to dispense precise amounts of multiple disease ligands such as antigens or antibodies or an analyte as a micro array onto a reaction membrane for use in the apparatus of the first aspect of the present invention. The ligands or analytes may be dispensed in particular patterns, e.g. letters for ease of recognition of results. Typically,
10 100 pl of fluid reagent (1 drop), or multiples thereof, is dispensed, but this will vary depending on the application. The resultant size of the spot on the membrane is about 55 microns or more in diameter subject to fluid diffusion on the membrane, but again this will vary depending on the application. It is possible to dispense droplets with diameters of 5-10 microns, and hence
15 lower volumes of fluid reagent (for example, 1-10 pl) can be applied. Using precise quantitative printing of micro arrays of antibodies, antigens, or other analytes means that tests using precise quantities of these reagents can be produced for multi disease diagnosis of a single sample. This array technology can be applied to tests for drugs or other markers across all
20 diagnostic fields.

 Alternatively, an adult/neonatal syringe pump 1235 from ATOM Medical Corporation, Japan, typically used to administer small quantities of intravenous liquids through a catheter to hospital patients can be adapted to apply single or multiple lines of a capture analyte to the first membrane eg
25 nitrocellulose.

 In one preferred embodiment, ligands for detecting tuberculosis, HIV, hepatitis, syphilis and malaria antibodies may be deposited onto a reaction membrane. This would allow the simultaneous diagnosis of tuberculosis, HIV, hepatitis, syphilis and malaria from a single blood sample without the
30 need for intermediate sample treatment steps.

 Utilising the present invention allows the assaying of small volumes of whole blood and thus the present invention provides a very rapid diagnostic assay device that is simple to use and can be used in both laboratory and point-of-care field diagnostic locations. For example, a finger prick of blood
35 would be sufficient to perform an assay. Similarly large volumes of sample can be used in this device by increasing the amount of absorbent material

(second member). For instance, 10 mls of dilute fluids like urine can be can be assayed to detect low abundance molecules.

Analytes and/or ligands (e.g. antigens or antibodies) can be printed down in titrating amounts and/or concentrations. Thus, in an individual
5 screen, this would provide a means of quantitating analyte-ligand levels within the sample solution.

The pre-incubation step may also be carried out with a multi-analyte detector where any number of detection analytes can be attached to a gold particle or a similar detectable tag e.g. a fluorescent marker.

10

Brief Description of the Drawings

A specific embodiment of the present invention will now be described by way of example only and with reference to the accompanying drawings in which:

15 Figure 1 is a schematic drawing of an apparatus embodying aspects of the present invention in a first configuration;

Figure 2 is a schematic drawing of the apparatus of Figure 1 in a second configuration;

20 Figure 3 is a perspective view of an assay apparatus or cassette embodying aspects of the present invention;

Figure 4 is an exploded view of the components of the cassette shown in Figure 3;

Figures 5a to 5d show various stages in the use of the apparatus of Figure 3 in carrying out an assay; and

25 Figure 6 is a graph comparing test results from samples spiked with alpha analyse undergoing no-pre-incubation with samples undergoing a one minute pre-incubation.

Brief Description of a Preferred Embodiment

30 Capture analytes in the form of ligands such as antigens or antibodies (e.g. TB, HIV-1) are printed onto a protein-capture membrane matrix (e.g. a nitrocellulose membrane) in an appropriately sized array using piezoelectric chemical printing technology. A suitable chemical printing system for use in the present invention involves the use of piezoelectric drop-on-demand ink
35 jet printing technology for micro-dispensing fluids in DNA diagnostics or the Combion Inc. synthesis process called "CHEM-JET". To explore drop on

demand fluid dispensing for DNA diagnostics, an eight fluid printer has been developed as part of the Genosensor Technology Development (GTD) project funded by the Institute of Standards and Technology (USA). Research to date, is focused on printing oligonucleotide micro-spots onto solid supports.

5 In the CHEM-JET technique, which was developed at the California Institute of Technology, tiny volumes of reagent bearing liquid are squirted onto specific spots or addresses of a solid substrate much as an ink-jet printer squirts ink onto a page. By repeatedly returning to each address with one or another of a small set of building blocks, in this case, nucleotides modified
10 for the process, huge two-dimensional libraries of short DNA chains (oligonucleotides) can be assembled. Such a device including an imaging means is described in the applicant's co-pending International patent application No PCT/AU98/00265, the entire contents of which are incorporated herein by reference. In the described embodiment, antigen is
15 printed onto a reaction membrane in 100 pl droplets, or multiples thereof (eg. 10 nl), with each aliquot being 1 mm apart. However, these volumes and distances can be increased/decreased accordingly depending on the chosen antigen titre and array size. For example, it is possible to dispense droplets with volumes as low as 1-10 pl.

20 In a particularly preferred embodiment, antigens or antibodies can be printed down in a matrix of dots or lines or in the shape of letters so that quantitative multiple analyte analysis of a single sample is possible.

After the dispensed antigen has dried, non-specific protein-binding sites on the (nitrocellulose) membrane are blocked using 0.5% (v/v) casein in
25 phosphate buffered saline (PBS) + 0.05% (w/v) sodium azide + 0.1% (v/v) Tween-20 (PBSA wash buffer). It is however an option to leave the membrane unblocked following the printing of the antigen (or antibody) or other ligand.

In another preferred embodiment syringe pump technology used for
30 the administration of liquids intravenously to patients can be adapted to lay down single or multiple lines on nitrocellulose membranes.

Turning to the drawings, Figure 1 shows a flow-through assay device
10, which utilises the nitrocellulose membrane described above. The device is in the form of a cassette 12 and an associated removable filter frame 14.
35 Inside the cassette there is the membrane (typically nitrocellulose) 16 on which capture analytes in the form of ligands are printed, as described above,

which is located on top of an absorbent matrix 18. The absorbent matrix preferably comprises multiple layers of absorbent tissue or an absorbent pad such as blotting paper, in the specific embodiment twenty-four layers (double ply), which have been found to possess an ideal porosity that permits the most rapid flow-through of various solutions. This rapid flow-through is important as it results in lower backgrounds with higher reaction specificity and higher signal resolution.

As shown in Figure 1, the top of the cassette defines an opening in its upper face and a depending generally frusto-conical well whose sides depend down as far as the membrane 16, to define a chamber having sloping sides and a base defined by the membrane 16.

The filter unit frame 14 is spaced above the upper surface of the cassette 12. It also defines a depending conical well in the form of a chamber 21 also referred to as a "pre-incubation chamber" having sloping sides and a base 22 formed from a 5µm Whatman grade 1 membrane or a 0.22 µm hydrophilic Durapore membrane filter (Millipore, North Ryde, Australia). However, other types of filter/membrane and pore size would be suitable depending on the application. The function of the membrane is to retain a sample to be assayed in the well or pre-incubation chamber 21 long enough for a "pre-incubation step" to take place. When membrane 22 is lowered to contact the membrane 16, capillary attraction draws the sample from the chamber 20 through membranes 22 and 16 and into the tissue 18.

For ease of use, two pins 24 are provided which support the filter frame 14 at an appropriate distance above the cassette 12 during the pre-incubation step but which allow the filter frame to be pushed down so that the membranes 22 and 16 are in contact for the second stage of the process shown in Figure 2. The frame 14 is also removable so that the membrane 16 can be viewed to determine the results of the assay.

Figures 3 to 5d illustrate one commercial assay device design embodying the aspects of Figures 1 and 2.

In those Figures, the components which are equivalent to components shown in Figures 1 and 2 carry the same reference numerals. The cassette 12 comprises an upper moulding 12a and a lower moulding 12b. The porous membrane 22 is defined by the base of a pressed filter paper frusto cone 22a held in place by a filter retainer 23. The filter unit frame 14 defines two

dimples 14a on which an operator's thumbs may press when depressing the filter frame to contact the membranes 22 and 16.

Figures 5a to 5d illustrate the stages of operation of the apparatus. Figure 5a illustrates the filter frame separate from the cassette 12. Figure 5b illustrates the pre-incubation positioned with the base of the chamber/well 21 spaced from membrane 16. Figures 5c and 5d illustrate the device after the filter unit has been pressed down to bring the membranes 22 and 16 into contact to allow the sample to flow through to the blotting paper 18.

If the membrane 22 is replaced with a hydrophobic membrane, it is possible to operate the device with a pre-incubation step solely in the position shown in Figures 3 and 4 with the membranes 22 and 16 always in contact. The hydrophobic membrane 22 will prevent flow of the sample in the incubation chamber 21 to the reaction membrane 16. After a sufficient period of time has past for detection analyte in the chamber 21 to bind to the reagent, a suitable wetting agent is added to the sample in the chamber which allows the sample to flow through the hydrophobic membrane past the reaction membrane 16 and into an absorbent matrix 20.

Example 1

Application of the pre-filter chamber

Whatman membrane (paper) or Reemay filters (polyester; 1cm²) are inserted into the chamber 21 in the filter frame to form a conical retaining vessel (pre-filter unit).

The sample is pipetted into the plastic pre-filter chamber (50-100 ul) along with a detection analyte in the form of a detecting antibody (50-100 ul) bound to colloidal gold (particle size 20-50 nm). The sample is pre-incubated with the gold-conjugate (O.D.4) within the pre-incubation chamber for thirty seconds after gentle pipetting to ensure adequate mixing. After thirty seconds the chamber is pressed into the well 20 of the test cassette 12. Upon contact with the membrane 16 containing the detection zone, the solution filters through to the absorbent layer 18 beneath. The pre-filter 14 is discarded when the solution has filtered through and two drops of PBSA wash buffer are then added to the reaction membrane to wash away excess gold-conjugate revealing the results of the assay on membrane 16.

The use of the pre-incubation of the sample with the detection analyte increases sensitivity by approximately ten fold. Further, any particulate

matter is retained in the pre-incubation chamber all of which can be removed to provide a clear signal. The use of the preincubation chamber with the dual roles of permitting a pre-incubation step and a pre-filtering step, also allows multi-analyte detection on the reaction membrane by pre-incubating with a
5 multi-analyte probe, e.g. colloidal gold bound to different detecting analytes. In addition, interfering analytes or substances that could cause false positives or negatives in the assay can be removed or absorbed out in the pre-incubation step, e.g. human antibodies to mouse antigens can be absorbed out by anti-HAMA antibodies.

10 Although the above described example relates to the antigens relating to disease, the immunoassay apparatus could be used, for example, as an allergy test kit, as a test kit for drugs of abuse or for analysing non-human derived samples e.g. bovine, porcine, veterinary tests, and tests in agriculture such as grain quality evaluation, etc.

15 The method and apparatus of the present invention is particularly suited to use with swabs which can be simply placed into the chamber 21, swirled around in liquid containing a detecting antibody (50-100 ul) bound to colloidal gold for 30 seconds before the pre-filter unit is depressed to contact the membranes 22 and 16 together.

20 Any combination of ligands and analytes can be applied to the system of the present invention. The choice of ligands could be tailored to detect prevalent diseases in a particular country or population. For example, analytes from the following combination of diseases could be used for diagnosis using this array.

25

1. TB and HIV
2. Hepatitis-B & C, HIV
3. Chagas, HIV, TB, Syphilis and Hepatitis-B & C
4. Malaria, Dengue, TB, Chagas.

30

Alternatively antigens representing different varieties of wheat or other agricultural products could be printed on the reaction membrane enabling detection of multiple strains with a single test.

Example 2

The assay device can also be used for detecting analytes in body fluids other than blood such as plasma, sera, urine, saliva and sputum. In this system, the sample can be retained in the pre-incubation chamber 22 by use
5 of a hydrophobic membrane such as Reemay or Hollingsworth and Vose 7303 instead of the Whatman grade 1 membrane or a 0.22 μ m hydrophilic Durapore membrane filter described above. The sample is mixed with the detection analyte for the required pre-incubation period. To obtain efficient flow through capillary action to the absorbent layer 18 when the pre-
10 incubation chamber 22 is lowered onto the cassette 12, one of two procedures can be followed:

1. The membrane 16 containing the capture analyte is pre-wet with at least one drop of wash buffer containing 0.01 M phosphate, 0.15 M NaCl, 0.0% Azide, 0.5% Tween 20 or any wetting agent containing a detergent;
- 15 2. The membrane 16 containing the capture analyte is blocked with a hygroscopic solution such as sucrose, trehalose, fructose, or alternatively, glycerol. This changes the characteristics of the membrane 16 from a non-hygroscopic to a hygroscopic membrane allowing the sample to flow through to the absorbent layer 18 upon contact of the membrane at the base of the pre-
20 incubation chamber 22 with membrane 16.

Example 3 (Comparative Example)

Comparison of no pre-incubation and 1 minute pre-incubation of a sample spiked with alpha amylase in the above described format.

25

Procedure

A 6% solution of bovine sera albumin was spiked with 0.1ng/ml, 0.5ng/ml, 1ng/ml, 10ng/ml, 50 ng/ml, 100ng/ml, 500ng/ml and 1000ng/ml and applied to the above format according to the following procedure:

30

No-preincubation

- I. The pre-incubation chamber was pressed down so that the base of the chamber comes into contact with the first member containing the capture antibody against alpha amylase.

- II. Sixty microlitres of 0.5% tween in saline was added to the pre-incubation chamber and allowed to filter through to the absorbent material beneath the first membrane.
- 5 III. One hundred microliters of spiked alpha amylase sample was added to the chamber and allowed to filter through to the absorbent material beneath the first membrane.
- IV. Sixty microlitres of 0.5% tween in saline was added to the pre-incubation chamber and allowed to filter through to the absorbent material beneath the first membrane.
- 10 V. Sixty microlitres of anti-alpha amylase antibody linked to colloidal gold (particle size 20-50nm) was added to the pre-incubation chamber and allowed to filter through to the absorbent material beneath the first membrane.
- VI. Sixty microlitres of 0.5% tween in saline was added to the pre-incubation chamber and allowed to filter through to the absorbent material beneath the first membrane.
- 15 VII. The pre-incubation chamber was removed and the result on the reaction membrane scanned with a densitometer. Signal strength was measured in pixel intensity.

20

One minute pre-incubation

- I. Sixty microliters of 0.5% tween in saline was added to first membrane and allowed to filter through to the absorbent material underneath.
- 25 II. The pre-incubation chamber was suspended over the first membrane so that there was a space between the chamber and the membrane.
- III. One hundred microliters of spiked alpha amylase sample and 60 microliters of anti-alpha amylase antibody linked to colloidal gold (particle size 20-50nm) were incubated in the pre-incubation chamber for 1 minute.
- 30 IV. The chamber was lowered until it came in contact with the first membrane and the mixture of sample and antibody-gold conjugate allowed to filter through to the absorbent material.
- V. Sixty microliters of 0.5% tween in saline was added to the pre-incubation chamber and allowed to filter through to the absorbent material.
- 35

VI. The pre-incubation chamber was removed and the result on the reaction membrane was scanned with a densitometer. Signal strength was measured in pixel intensity.

Each data point on the graph is the average of two experiments using the apparatus described above. The results show that pre-incubation of the sample with the detection analyte has a minimal detection limit defined in pixel density of around 500 pg/ml of alpha amylase. This is compared to a minimum detection limit without the pre-incubation of about 50ng/ml and indicates the pre-cubation increases the sensitive by around 10 fold.

Example 4 (Comparative Examples)

Demonstration of increased sensitivity with increased pre-incubation of the sample with the detection analyte.

Samples of amylase diluted in 0.5% saline to 400 ng/mL were treated with immunogold conjugate against amylase and aliquotted onto the flow-through format in different protocols as shown below.

- A. The sample was added to the format (without a filter present) and allowed to filter through prior to adding conjugate, followed by an aliquot of conjugate immediately the sample had passed through the membrane.
- B. The sample was mixed in the correct proportions with gold conjugate and aliquotted immediately onto the flow-through format.
- C. The sample was mixed as with protocol B but added to the flow through format after a 60 second interval.

The results presented in pixel intensity are shown in the tables below (for 2 experiments):

Protocol	Sample peak	Control peak	Sample area	Control area	S/PC ratio
A	82	286	657	2120	317
B	288	758	2062	5509	383
C	823	949	5843	6765	884

Protocol	Sample peak	Control peak	Sample area	Control area	S/PC ratio
A	89	516	588	3890	588
B	482	830	3736	6345	602
C	708	829	4506	5822	792

Clearly there is a significant increase in the sample signal when the analyte is preincubated with the conjugate probe, as distinct to sequential
5 detection on the flow-through format. The difference in detection levels (for the 400 ng/mL sample) equated to between a 7.5-fold to 10-fold increase in detectable amylase in the flow through format when the sample is preincubated separately to the detecting capture antibody.

It will be appreciated by persons skilled in the art that numerous
10 variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. An apparatus for use in an assay process comprising:
a first member comprising a first, porous, membrane to which is bound
a capture analyte for binding to a reagent to be detected, the member having
5 an upper surface and a lower surface;
a second member being a body of absorbent material such as tissue
paper or the like disposed below and touching the lower surface of the first
member;
a chamber spaced above the first member said chamber having side
10 walls and a base defined by a second membrane; and
means for supporting the chamber above the first member in two
positions, a first position in which the membrane is spaced a sufficient
distance from the first member so as to not permit fluid transfer from the
chamber to the body of absorbent material, and a second position in which
15 the membrane is in contact with the first member thus permitting fluid
transfer from the chamber through the first and second membranes to the
body of absorbent material.
2. An apparatus as claimed in claim 1 wherein the second membrane
defined at the base of the chamber is a hydrophilic membrane.
- 20 3. An apparatus as claimed in claim 1 wherein the second membrane
defined at the base of the chamber is a hydrophobic membrane.
4. An apparatus as claimed in claim 2 wherein the first membrane is a
nitrocellulose membrane.
5. An apparatus as claimed in any preceding claim wherein the capture
25 analyte is a ligand such as an antigen or antibody.
6. An apparatus for use in an assay process comprising a housing
including:
a first member comprising a first, porous, membrane to which is bound
a capture analyte for binding to a reagent to be detected, the member having
30 an upper surface and a lower surface;
a second member being a body of absorbent material such as tissue
paper or the like disposed below and touching the lower surface of the first
member;
a chamber located above the first member said chamber having side
35 walls, and a base including a second, hydrophobic, membrane, having an
upper and a lower surface, the pre-incubation chamber being supported

above the first member with the lower surface of the hydrophobic membrane in contact with the upper surface of the first member.

7. An apparatus as claimed in any preceding claim wherein the chamber is a well.

5 8. An apparatus as claimed in any preceding claim wherein the capture analyte is a ligand such as an antigen or antibody.

9. An apparatus as claimed in any preceding claim wherein the body of absorbent material comprises multiple layers of absorbent tissue.

10. A method for assaying for the presence of a pre-determined reagent
10 comprising the steps of:

a) providing a first porous membrane to which capture analytes for binding to the reagent have been bound;

b) placing a sample to be assayed and a detection analyte in a chamber having a base defined by a second porous membrane;

15 c) allowing a sufficient period of time to pass for the detection analyte to bind to the reagent, if present;

d) contacting the base of the chamber with the first porous membrane; and

20 e) causing the sample to flow through the membranes to allow the reagent to bind to the capture analyte carried on the first membrane.

11. A method as claimed in claim 10 further comprising the steps of:
removing the chamber and washing the first membrane with a buffer prior to inspecting the membrane for the presence of the detection analyte.

12. A method as claimed in claim 10 further comprising the steps of:
25 washing the first membrane with a buffer by addition to the chamber before removal and inspection of the first membrane for the presence of the detection analyte.

13. A method for assaying for the presence of a pre-determined reagent using an apparatus as claimed in any one of claims 1 to 9 comprising the
30 steps of:

a) placing a sample to be assayed and a detection analyte in the chamber, with the chamber disposed in the first position;

b) allowing a sufficient period of time to pass for the detection analyte to bind to the reagent, if present;

35 c) depressing the chamber to the second position to contact the base of the chamber with the first porous membrane;

d) allowing the sample to flow through the first and second membranes to allow the reagent, if present to bind to the capture analyte carried on the first membrane.

14. A method as claimed in claim 13 wherein the second membrane
5 defined at the base of the chamber is hydrophobic and the step of allowing the sample to flow through the first and second membranes includes the addition of a wetting agent on the upper surface of the second membrane prior to moving the chamber to the second position.

15. A method for assaying for the presence of a pre-determined reagent
10 using an apparatus as claimed in claim 3 or in any one of claims 7 to 9 comprising the steps of:

a) placing a sample to be assayed and a detection analyte in the chamber;

b) allowing a sufficient period of time to pass for the detection
15 analyte to bind to the reagent, if present;

c) adding a wetting agent to the chamber;

d) allowing the sample to flow through the first and second porous membranes to allow the reagent, if present to bind to the capture analyte carried on the first membrane.

20 16. A method as claimed in any one of claims 10 to 15 wherein the sample is whole blood wherein whole red blood cells are removed in the chamber by the second membrane acting as a filter and plasma in the blood is allowed to flow-through to the first membrane.

17. A method as claimed in any one of claims 10 to 15 wherein the sample
25 contains particulate materials selected from the group including grain extracts, cell or microbial extracts wherein particulate materials are removed in the chamber by the second membrane acting as a filter.

18. A method as claimed in any one of claims 10 to 15 wherein the sample
30 comprises body fluids such as plasma, sera, urine, saliva and sputum and wherein the second membrane is a hydrophobic membrane.

19. A method as claimed in any one of claims 10 to 15 including the step of removing or neutralising unwanted analytes in the sample to be tested that may interfere with the binding of the sample analyte with the capture analyte on the first membrane in the chamber.

20. A method as claimed in claim 19 wherein antibodies or other analytes which bind to the unwanted analytes are bound to the walls or the base of the chamber.

21. A method as claimed in any one of claims 10 to 15 wherein the sample
5 to be tested is carried on an absorbent surface such as a swab or the like and including the steps of swirling the swab in an extraction solution in the chamber.

22. Apparatus as claimed in any one of claims 1 to 9 wherein the capture analytes comprise ligands selected from the group consisting of ligands for
10 detecting tuberculosis, ligands for detecting HIV, ligands for detecting hepatitis, ligands for detecting syphilis and ligands for detecting malaria.

23. Apparatus as claimed in claim 22 wherein the first membrane carries capture analytes for detecting more than one disease.

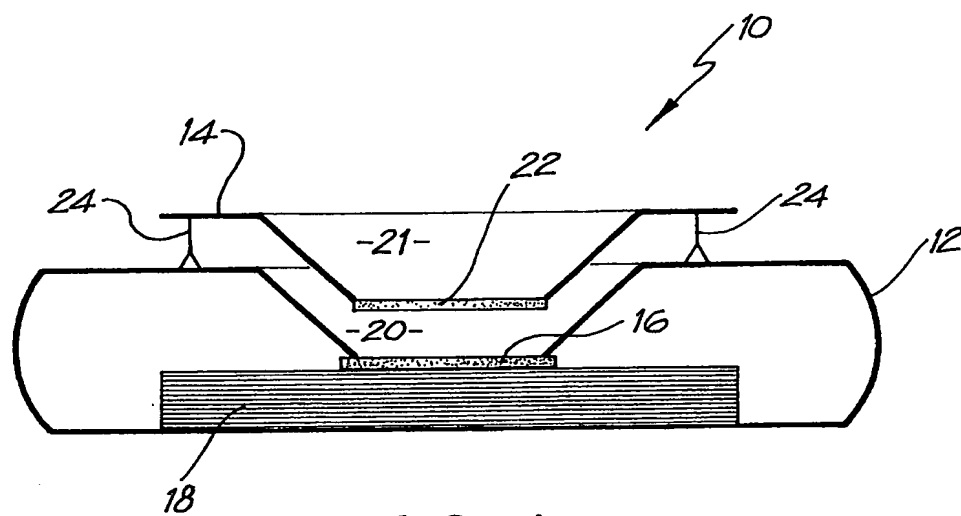


FIG. 1

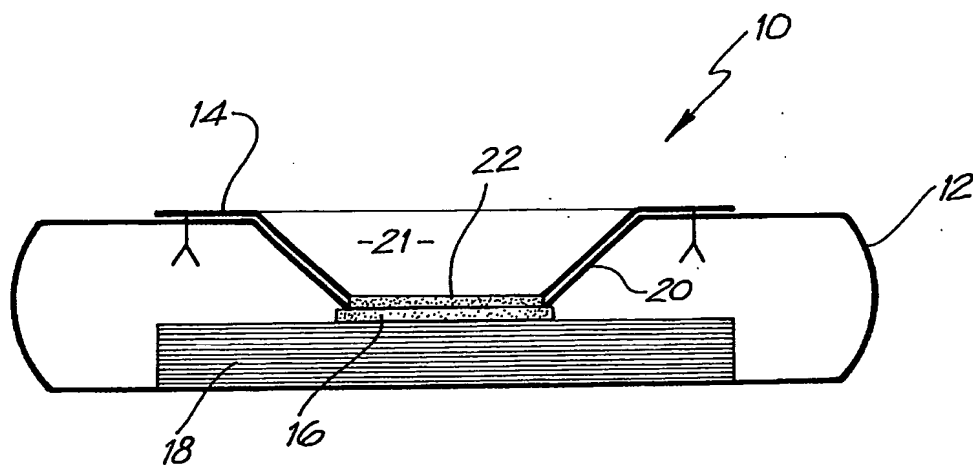
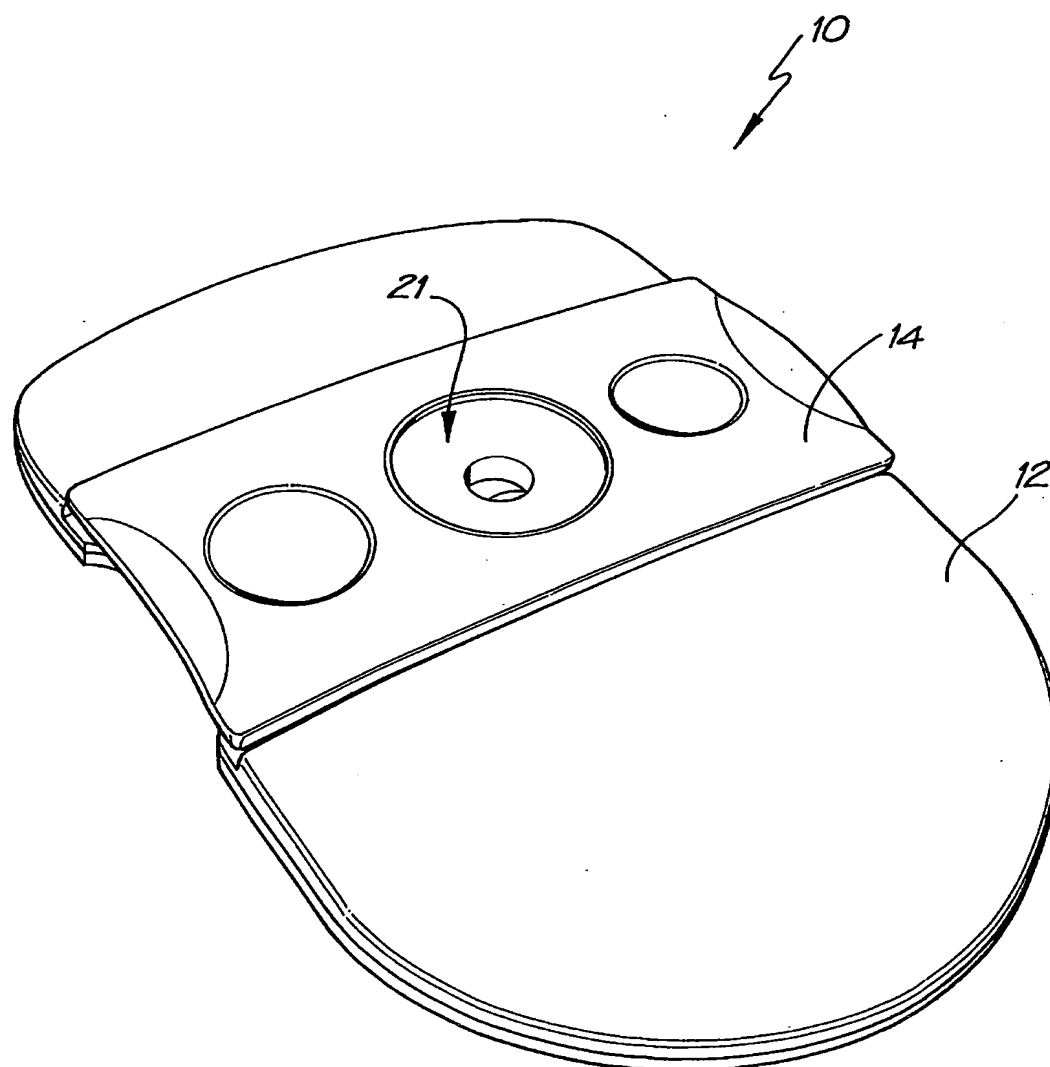
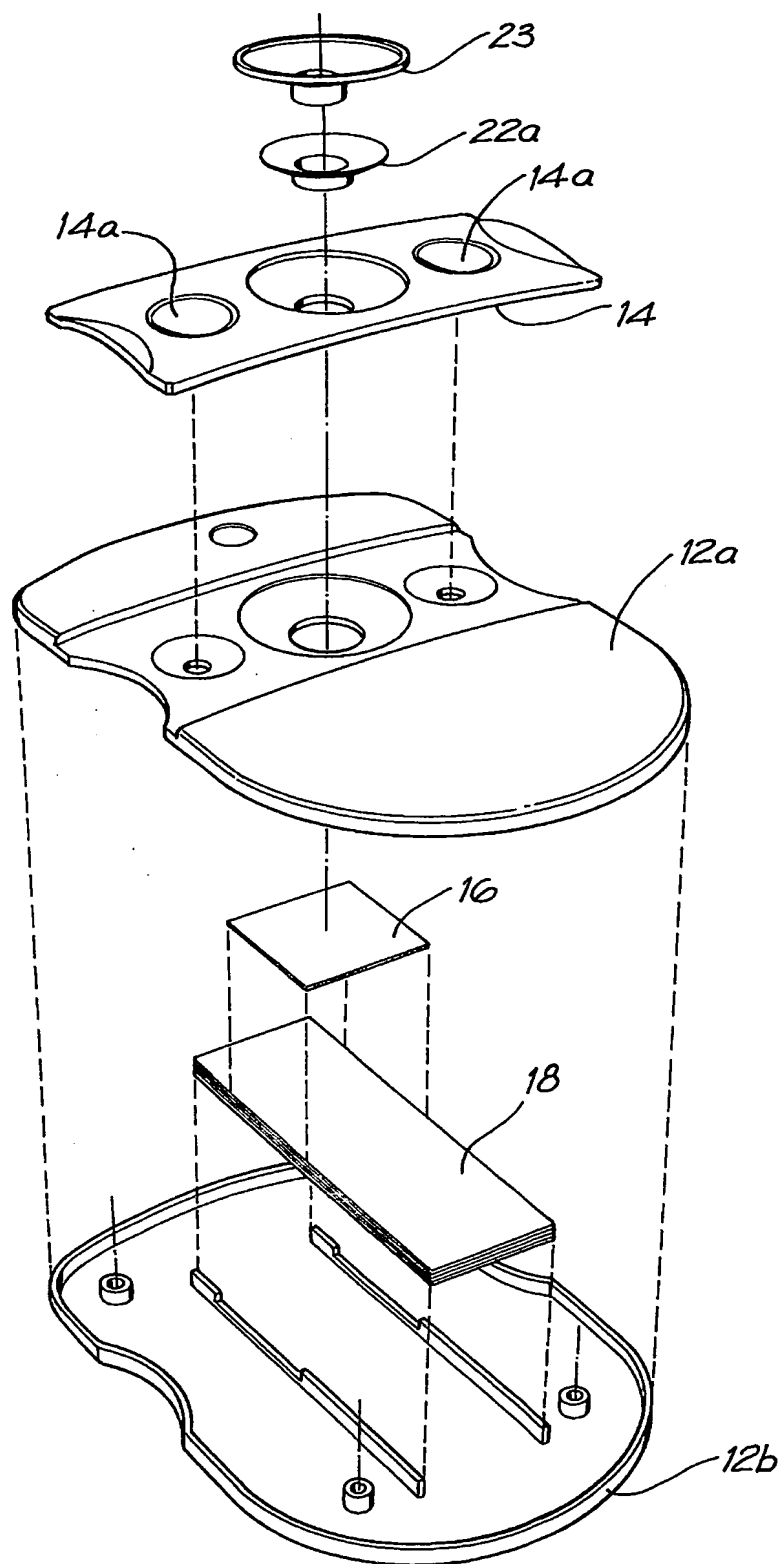


FIG. 2

**FIG. 3**

**FIG. 4**

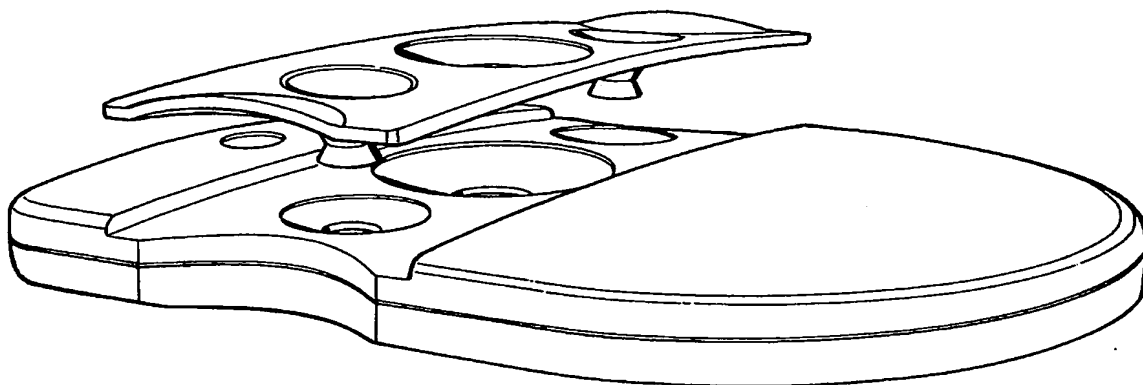


FIG. 5a

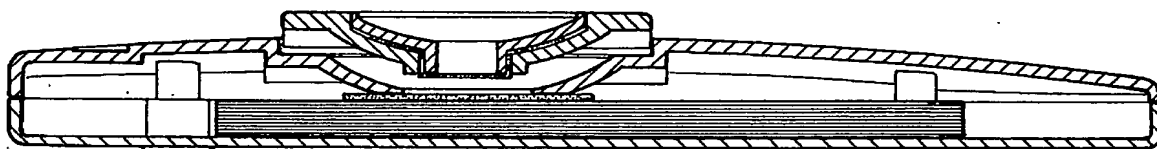


FIG. 5b



FIG. 5c

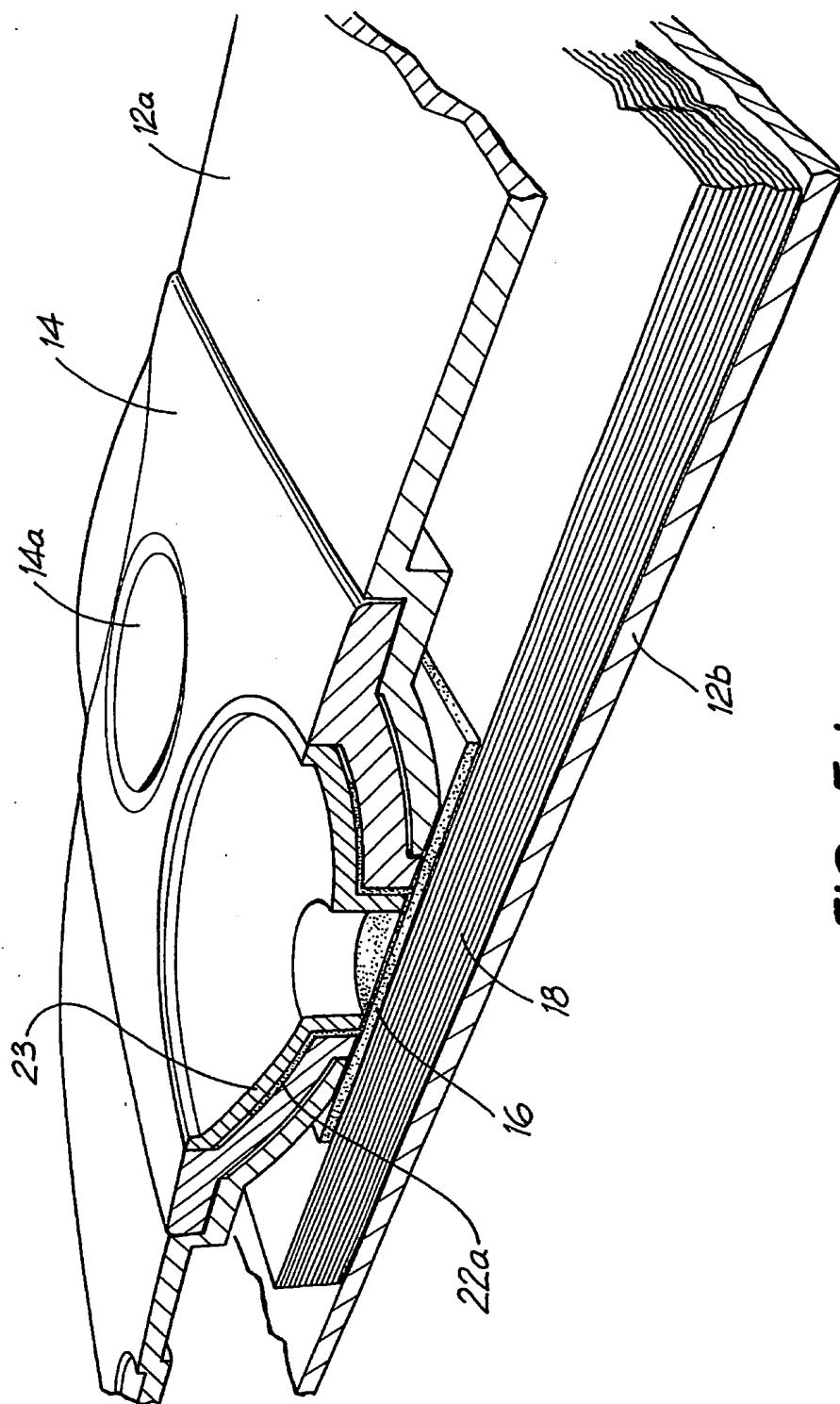


FIG. 5d

COMPARISON BETWEEN NO PRE-INCUBATION AND A ONE
MINUTE PRE-INCUBATION OF THE SAMPLE WITH THE
DETECTION ANTIBODY

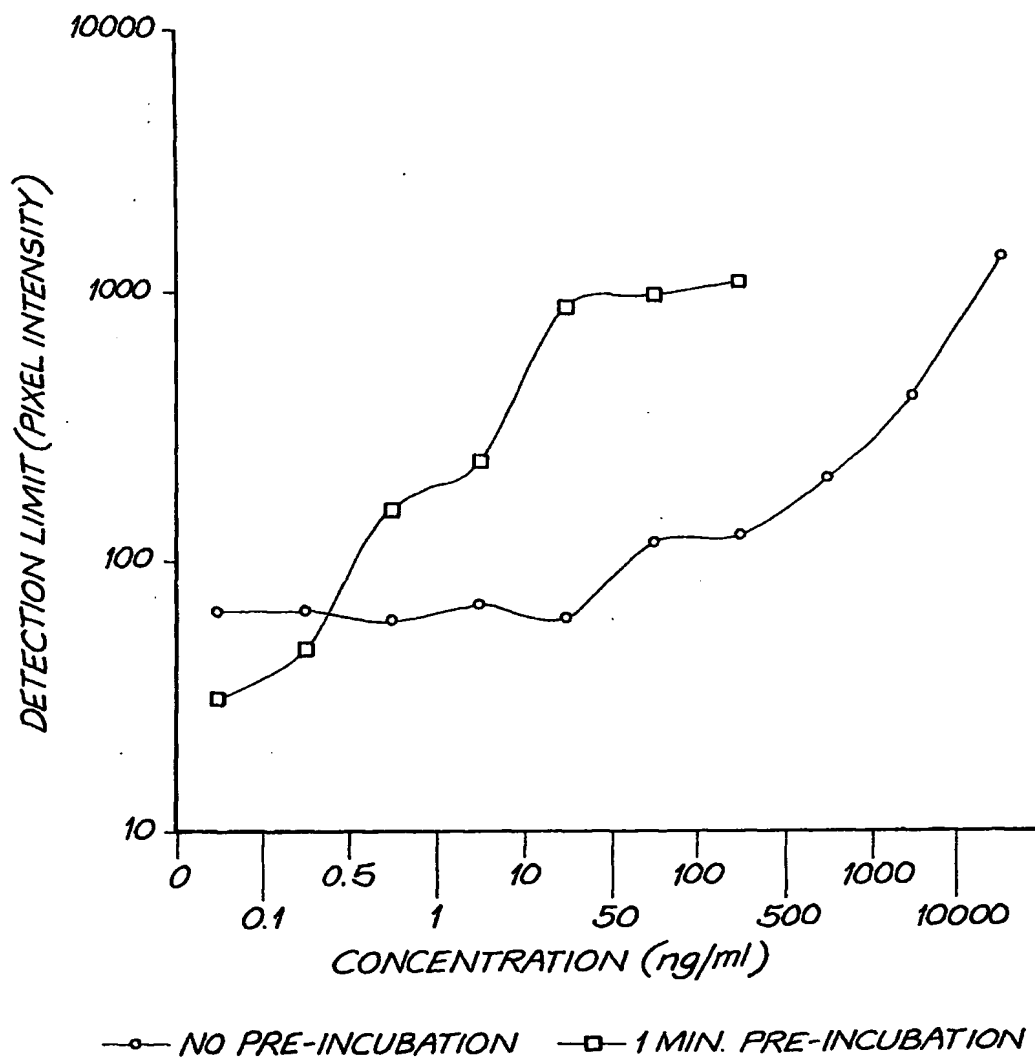


FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/01119

A. CLASSIFICATION OF SUBJECT MATTER		
Int. Cl. ⁷ : G01N 33/52, 33/53, 33/536, 33/543, 33/571, 33/576, 35/00, 35/10; C12M 1/34		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) Derwent WPAT, Japio; STN CAPLUS, MEDLINE, BIOSIS; Keywords.		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X, Y	WO 02/31465 A1 (INHALIX PTY LTD) 18 April 2002; see the figures in particular.	1-23
X,Y	WO 99/36773 A1 (BELLON PTY LTD; THE INSTITUTE OF RESPIRATORY MEDICINE) 22 July 1999; See the figures and examples in particular.	1-23
X,Y	WO 96/07099 A1 (THE UNIVERSITY OF SYDNEY) 7 March 1996; see the figures and examples in particular.	1-23
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C <input checked="" type="checkbox"/> See patent family annex		
<p>* Special categories of cited documents:</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier application or patent but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&" document member of the same patent family</p>		
Date of the actual completion of the international search 15 November 2002		Date of mailing of the international search report 25 NOV 2002
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustalia.gov.au Facsimile No. (02) 6285 3929		Authorized officer DAVID HENNESSY Telephone No : (02) 6283 2255

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU02/01119

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 01/84153 A2 (IDEXX LABORATORIES, INC.) 8 November 2001; see the figures in particular.	1-23
Y	US 5885526 A (CHU) 23 March 1999; see the figures in particular.	1-23
Y	US 6057165 A (MANSOUR) 2 May 2000; see the figures in particular.	1-23
A	WO 99/64863 A1 (NEW HORIZON DIAGNOSTICS INC.) 16 December 1999; see the figures and examples.	1-23
A	WO 98/32018 A1 (CARBURY HERNE LIMITED) 23 July 1998; see the figures and examples.	1-23
Y	EP 0447154 B1 (BIOSITE DIAGNOSTICS INC.) 26 January 2000; see the figures and examples.	1-23
A	WO 96/35123 A1 (CARTER WALLACE INC.) 7 November 1996; see the figures and examples.	1-23
A	FOOD AND AGRICULTURE IMMUNOLOGY, vol. 12, 31-40; Verheijen, R. et al. (2000) Development of a one step strip test for the detection of (dihydro)streptomycin residues in raw milk; see the abstract and figures in particular.	1-23

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU02/01119

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	02/31465	AU	20000748	AU	200195268
		WO	2002059569	AU	20012719
WO	99/36773	AU	1310/98	AU	20403/99
WO	96/07099	AU	7658/94	AU	33348/95
		EP	777858	JP	10505418
WO	01/84153	AU	200153354	US	6436722
US	5885526	CN	1256754	EP	988546
		WO	9843083	JP	2001525063
US	6057165	NONE			
WO	99/64863	AU	45625/99	EP	1086375
WO	98/32018	AU	55704/98	EP	968426
		JP	2001508180	GB	9700759
EP	447154	AT	189309	AU	74650/91
		CA	2037963	DE	69131933
		IE	910804	IE	70333
		JP	3070764	PT	97000
		US	5922615	US	6297060
WO	96/35123	AU	58521/96	AU	709403
		EP	823972	JP	10511774
				CA	2218995
				US	6319676
END OF ANNEX					